

**EXHIBIT I**

*Molecular Immunology*, Vol. 30, No. 16, pp. 1443-1453, 1993  
Printed in Great Britain.

0161-5890/93 \$6.00 + 0.00  
© 1993 Pergamon Press Ltd

## CONSTRUCTION AND INITIAL CHARACTERIZATION OF A MOUSE-HUMAN CHIMERIC ANTI-TNF ANTIBODY

DAVID M. KNIGHT,\* HAN TRINH,\* JUNMING LE,† SCOTT SIEGEL,‡ DAVID SHEALY,‡  
MARGARET McDONOUGH,\* BERNARD SCALLON,\* MARIA AREVALO MOORE,\* JAN VILCEK,†  
PETER DADDONA,‡ and JOHN GHRAYEB\*

Departments of \*Molecular Biology and †Immunobiology, Centocor, 200 Great Valley Parkway,  
Malvern, PA 19355, U.S.A.; and ‡Department of Microbiology and Kaplan Cancer Center, New  
York University Medical Center, New York, NY 10016, U.S.A.

*(First received 5 April 1993; accepted in revised form 22 July 1993)*

**Abstract**—Tumor necrosis factor- $\alpha$  (TNF) has been implicated in the pathogenesis of a variety of human diseases including septic shock, cachexia, graft-versus-host disease and several autoimmune diseases. Monoclonal antibodies directed against TNF provide an attractive mode of therapeutic intervention in these diseases. We have generated a murine monoclonal antibody (A2) with high affinity and specificity for recombinant and natural human TNF. To increase its therapeutic usefulness, we used genetic engineering techniques to replace the murine constant regions with human counterparts while retaining the murine antigen binding regions. The resulting mouse-human chimeric antibody should have reduced immunogenicity and improved pharmacokinetics in humans. Molecular analysis of light chain genomic clones derived from the murine hybridoma suggests that two different alleles of the same variable region gene have rearranged independently and coexist in the same hybridoma cell. The chimeric A2 antibody (cA2) exhibits better binding and neutralizing characteristics than the murine A2 which was shown to contain a mixture of two kappa light chains. The properties of cA2 suggest that it will have advantages over existing murine anti-TNF antibodies for clinical use.

### INTRODUCTION

The cytokine tumor necrosis factor- $\alpha$  (TNF) has been implicated as a crucial mediator in the pathogenesis of infectious, neoplastic and autoimmune diseases. Its role in the regulation of inflammatory and immunological responses makes it an attractive target for therapeutic intervention in a variety of human diseases by neutralization of its action. For example, elevated levels of TNF have been implicated in the profound weight loss (cachexia) associated with chronic parasitic or bacterial diseases and cancer, the circulatory collapse and shock resulting from acute bacterial infection, the development of graft-versus-host disease and the pathogenesis of rheumatoid arthritis [for reviews see Beutler and Cerami (1988), Tracey and Cerami (1989), Brennan *et al.* (1991)].

Animal models have reinforced the notion that TNF is involved in gram-negative (Shimamoto *et al.*, 1988; Opal *et al.*, 1990) and gram-positive (Freudentberg and Galanos, 1991) bacteremia, septic shock (Tracey *et al.*, 1987; Silva *et al.*, 1990; Hinshaw *et al.*, 1990), bacterial meningitis (Leist *et al.*, 1988), autoimmune lupus (Jacob and McDevitt, 1988), graft-versus-host disease (Shalaby *et al.*, 1989) and multiple sclerosis (Hofman *et al.*, 1989). These studies have suggested that anti-TNF monoclonal antibodies (mAbs) may have therapeutic potential for a wide variety of human diseases.

Of particular interest is the potential use of anti-TNF mAbs for the treatment of the widespread inflammatory disease rheumatoid arthritis. Elevated levels of TNF and

its receptors have been found in the sera, synovial fluid and tissues of patients with rheumatoid arthritis (Cope *et al.*, 1992; Deleuran *et al.*, 1992), and high levels of TNF are secreted by synovial cells in culture (Brennan *et al.*, 1989a). In addition, antibodies directed against TNF have been shown to inhibit production of the pro-inflammatory cytokine IL-1 in synovial cell cultures from patients with rheumatoid arthritis, suggesting that TNF may be the main inducer of IL-1 and that anti-TNF antibodies may be useful in the treatment of this disease (Brennan *et al.*, 1989b). In mice with collagen-induced arthritis, a hamster anti-TNF mAb was able to reduce the severity of the disease even when injected after the onset of clinical arthritis (Williams *et al.*, 1992).

To date, there have been little published data available on the use of anti-TNF mAb therapy in humans. Murine anti-TNF mAbs have been administered to patients with severe septic shock (Exley *et al.*, 1990; Spooner *et al.*, 1991) but the limited scope of these Phase I studies did not permit evaluation for efficacy. It is rapidly becoming axiomatic that modification of murine mAbs to a more human form is desirable when therapeutic use is envisioned. Murine antibodies, as foreign proteins, may elicit immune reactions that reduce or eliminate their therapeutic efficacy and/or evoke allergic or hypersensitivity reactions in patients upon readministration with the same antibody or even an unrelated murine mAb. Chimeric antibodies consisting of murine antigen binding regions joined to human constant regions have been suggested as a means to reduce the immunogenicity of

Best Available Copy

murine antibodies. Although there is limited data comparing the immunogenicity of murine and chimeric antibodies in humans, initial reports suggest that chimeric antibodies are less immunogenic than their murine counterparts (LoBuglio *et al.*, 1989; Meredith *et al.*, 1991; Saleh *et al.*, 1992). Here we describe the construction and initial characterization of a chimeric anti-TNF antibody with improved binding characteristics compared to the reagent derived from the murine hybridoma. This chimeric antibody (cA2) has been used in Phase I clinical trials in patients with severe rheumatoid arthritis (Elliott *et al.*, 1993) and shows exceptional promise as a therapeutic agent in this disease.

#### MATERIALS AND METHODS

##### *Generation of the murine A2 hybridoma*

A BALB/c mouse was injected intraperitoneally with 10 µg of purified recombinant human TNF (rTNF) emulsified with an equal volume of complete Freund's adjuvant. An injection of 5 µg of rTNF with incomplete Freund's adjuvant followed by three consecutive injections of 5 µg of rTNF without adjuvant were given at weekly intervals starting 1 week after the first immunization. The mouse was boosted intraperitoneally 4 weeks later with 10 µg of rTNF. Four days after the last injection the mouse was sacrificed, and the spleen cells were fused with the hybridoma SP2/0 (from Memorial Sloan-Kettering Cancer Center) at a 4:1 ratio of spleen cells to SP2/0 cells, with 0.3 ml of 30% PEG 1450. After incubation at 37°C for 6 hr, the fused cells were plated at a density of  $2 \times 10^4$  cells/well in 96-well mitrotiter plates in the presence of  $5 \times 10^4$  feeder spleen cells from a normal BALB/c mouse. The growth medium was RPMI 1640, 10% heat-inactivated fetal bovine serum (Hyclone), 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and HAT (Boehringer Mannheim). A solid-phase radioimmunoassay was employed for screening monoclonal antibodies specific for human TNF. Positive wells were initially subcloned at limiting dilution on mouse feeder cells, and subsequently further subcloned in the absence of feeder cells. The hybridoma line A2, which secretes a murine IgG1 antibody was chosen for further studies. Antibody from A2 cell supernatant was purified by protein A-sepharose.

##### *Genomic library construction*

To isolate the light chain variable region gene from the A2 hybridoma, a size-selected genomic library was constructed using the phage  $\lambda$  vector charon 27. High molecular weight DNA was isolated from A2 hybridoma cells and digested to completion with restriction endonuclease *Hind* III. The DNA was then fractionated on a 0.8% agarose gel and three size classes of fragments, 3, 4 and 6 kb, were isolated from the gel by electroelution. These sizes were chosen based upon bands which hybridized to the  $J_{\kappa}$  probe on a Southern blot. After phenol/chloroform extraction and ethanol precipitation,

the *Hind* III fragments were ligated with  $\lambda$  charon 27 arms and packaged into phage particles *in vitro* using Gigapack Gold from Stratagene. The libraries were screened directly at a density of approximately 20,000 plaques per 150 mm petri dish using a  $^{32}P$ -labeled  $J_{\kappa}$  probe. Plaque hybridizations were carried out in  $5 \times SSC$ , 50% formamide, 2 × Denhardt's reagent, 200 µg/ml denatured salmon sperm DNA at 42°C for 18–20 hr. Final washes were in  $0.5 \times SSC$ , 0.1% SDS at 65°C. Positive clones were identified after autoradiography.

To isolate the variable region gene for the A2 heavy chain, a genomic library was constructed in the lambda vector  $\lambda$ gt-10. High molecular weight DNA was digested to completion with restriction endonuclease *Eco* R I and fragments of approximately 7.5 kb (corresponding to a hybridoma-specific band on a Southern blot) were isolated after agarose gel electrophoresis. These fragments were ligated with  $\lambda$ gt-10 arms and packaged into phage particles *in vitro* using Gigapack Gold. Screening, hybridization and wash conditions were identical to those used for the light chain library, except that the  $J_{\kappa}$  probe was used.

##### *DNA probes*

The mouse heavy chain  $J_{\kappa}$  probe was a 2 kb *Bam*H I/*Eco* R I fragment containing both  $J_3$  and  $J_4$  segments (Fig. 1A). The mouse light chain  $J_{\kappa}$  probe was a 2.7 kb *Hind* III fragment containing all five  $J_{\kappa}$  segments (Fig. 1B).  $^{32}P$ -labeled probes were prepared by random priming using a kit obtained from Boehringer Mannheim. Free nucleotides were removed by centrifugation through a Sephadex G-50 column. The specific activities of the probes were approximately  $10^9$  cpm/µg.

##### *Transfection of cells by electroporation*

Plasmid DNA containing the cloned mouse V genes and human constant region genes was purified by centrifuging to equilibrium in ethidium bromide–cesium chloride gradients twice. Plasmid DNA (10–50 µg) was added to  $10^7$  SP2/0 cells (obtained from S. Warnaar) in Hanks' salts medium and the mixture exposed to 200 V and 960 µF in a Biorad electroporation apparatus. Cells were plated out in 96-well mitrotiter plates. SP2/0 cells were grown in Iscove's medium supplemented with 5% fetal bovine serum. Selective medium included 0.5 µg/ml mycophenolic acid, 50 µg/ml xanthine and 2.5 µg/ml hypoxanthine. Selection was applied after 48 hr and drug-resistant colonies were identified after 1–2 weeks. Individual transfectants were analysed for IgG production by ELISA and the highest producer was subcloned at least twice by limiting dilution.

##### *Quantitation of antibody production*

Tissue culture supernatant was analysed for IgG protein content by ELISA assay using standard curves generated with purified IgG. The concentration of chimeric A2 antibody with human constant regions was determined using goat anti-human IgG Fc antibody-coated microtiter plates and alkaline phosphatase-conju-

## Characterization of a mouse-human chimeric anti-TNF antibody

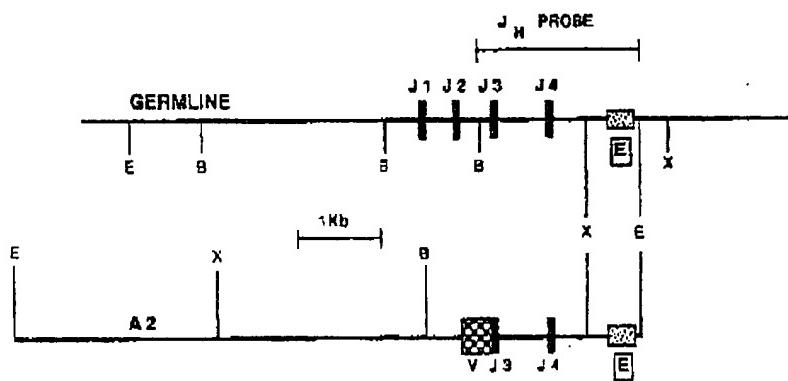
1445

gated goat anti-human IgG Fc or goat anti-human IgG (H + L) antibody. Coated plates were washed three times with Tris-buffered saline, 0.05% Tween-20 (TBS/Tween) and samples were added (50 µl/well). Plates were incubated for 30 min at room temperature followed by three washes with TBS/Tween. Conjugated antibody was added and the plates were incubated for 30 min at room temperature, followed by four washes with TBS/Tween. Substrate was prepared by dissolving two phosphatase substrate tablets (Sigma 104) per 10 ml of alkaline buffer solution (Sigma 221) diluted 1:500 in sterile saline. Substrate was added (100 µl) and the plates were incubated for 15 min at room temperature. The reactions were stopped by the addition of 50 µl/well of 3 N NaOH, and the absorbance was determined at 410 nm using a Dynatech plate reader.

*TNF solid-phase ELISA*

Round bottom, 96-well polyvinylchloride plates (Costar) were coated with rTNF (Dainippon) at 2 µg/ml in phosphate-buffered saline pH 7.2 (PBS) by incubating 40 µl/well overnight at 4°C. The wells were washed three times with PBS, then blocked with 150 µl/well of PBS containing 2% bovine serum albumin (BSA) by incubating for 1 hr at 37°C. Serial dilutions of the test antibody and any control antibodies were prepared in PBS-2% BSA. The blocked plate was washed three times with PBS, then each dilution was dispensed 50 µl/well in triplicate. After incubation for 1 hr at 37°C the plate was

A.



B.

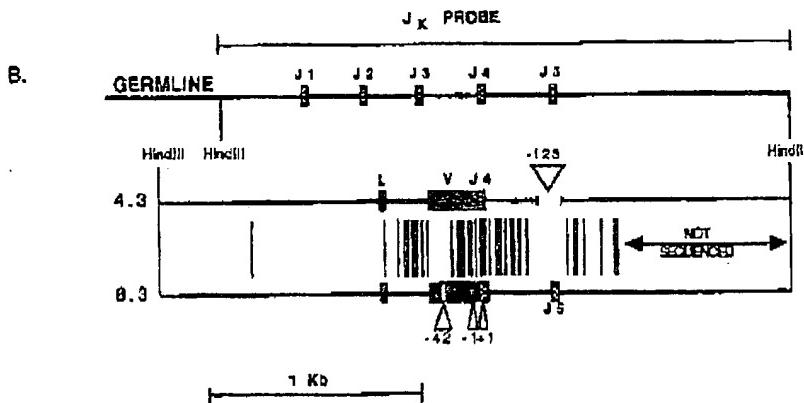


Fig. 1. Restriction enzyme maps of heavy and light chain clones derived from the A2 hybridoma, and the germline *J<sub>H</sub>* and *J<sub>K</sub>* loci. A. Heavy chain locus and A2 heavy chain clone. *J<sub>H</sub>* segments 1-4 are shown, along with the heavy chain enhancer (E) and the A2 heavy chain variable region (V). The location of the *J<sub>H</sub>* probe is indicated. B. *Bam*H I; E, *Eco*R I; X, *Xba*I. B. Maps of the *J<sub>K</sub>* locus and the 4.3 and 8.3 genomic clones from the A2 hybridoma. *J<sub>K</sub>* segments 1-5 are shown along with the location of the *J<sub>K</sub>* probe. The leader peptide (L) and light chain variable region (V) are shown. Vertical lines between the maps of 4.3 and 8.3 represent single base pair differences between the two clones. Deletions and insertions are indicated by triangles.

again washed three times in PBS. For murine antibody samples, a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (H&L) (Jackson Immunoresearch Labs) was prepared in PBS-2% BSA, whereas for chimeric antibodies a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-human IgG (H&L) (Jackson Immunoresearch Labs) in PBS-2% BSA was prepared. The appropriate conjugate was dispensed 50  $\mu$ l/well and incubated for 1 hr at 37°C. Substrate was prepared by dissolving one phosphatase substrate tablet (Sigma 104) per 5 ml of alkaline buffer solution (Sigma 221) diluted 1:500 in deionized water. The plate was washed three times with PBS, then substrate dispensed 50  $\mu$ l/well and incubation continued for 20 min at room temperature. Color development was stopped by the addition of 50  $\mu$ l of 3 N NaOH to each well. The absorbance of each well was then read at 410 nm, replicate wells were averaged and a plot of antibody concentration vs optical density at 410 nm prepared.

#### *Radioiodination of antibodies*

Microfuge tubes (1.5 ml) were coated with iodogen (1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycouril, Pierce), dissolved at a concentration of 5  $\mu$ g/50  $\mu$ l of methylene chloride, by dispensing 50  $\mu$ l per tube and allowing the solvent to evaporate in a fume hood. These tubes were then stored at -20°C. Fifty  $\mu$ g of antibody to be iodinated was diluted in 0.1 M sodium phosphate pH 7.0 to a volume of 50  $\mu$ l and transferred to an iodogen-coated microfuge tube. Immediately, 0.5 mCi (2.5  $\mu$ l) of carrier-free Na<sup>125</sup>I (Amersham) was added and the mixture was allowed to incubate for 6 min at room temperature. The reaction was quenched by adding 500  $\mu$ l of 0.1 M phosphate pH 7.0 containing 20 mg/ml potassium iodide and 0.83 mg/ml of ascorbic acid and the mixture was loaded onto a PD-10 column (Pharmacia) equilibrated in 0.1 M phosphate pH 7.0 containing 0.2% BSA and 0.1% sodium azide. The column was eluted with the same buffer and six 1 ml fractions collected and counted in a dose calibrator set for <sup>125</sup>I. The peak fractions (3 and 4) were pooled and the specific activity was calculated. The <sup>125</sup>I-labeled antibody was stored at 4°C.

#### *Relative affinity determination*

Ninety-six well assay plates were coated with TNF and blocked as described for the ELISA assay. Serial two-fold dilutions of the <sup>125</sup>I-antibody samples were prepared in PBS-2% BSA, beginning at 1.0  $\mu$ g/ml, and triplicate aliquots of each dilution were dispensed onto the plate, 50  $\mu$ l/well. After incubating for 1 hr at 37°C, the plate was washed three times in PBS and the wells cut out and counted in a gamma counter set for <sup>125</sup>I. Replicate wells were averaged, and the results used to calculate the nanograms (ng) of antibody bound to the well and the molar concentration of free antibody. A plot of ng bound vs ng bound/[free] was prepared and the slope calculated by fitting a straight line to the

points. This slope is an estimate of the relative association constant (Ka) for the antibody.

#### *Competitive inhibition assay*

Competitive binding between murine and chimeric A2 was demonstrated using <sup>125</sup>I-A2 and <sup>125</sup>I-cA2. Plates were coated with TNF and blocked as described previously. Serial two-fold dilutions of unlabeled competitor antibodies were prepared in PBS-2% BSA. The antibody dilutions were mixed with an equal volume of <sup>125</sup>I-A2 (50 ng/1,270,000 dpm/ml), or <sup>125</sup>I-cA2 (50 ng/1,400,000 dpm/ml). Fifty microliter aliquots were dispensed in duplicate onto a coated plate and incubated for 1 hr at 37°C. The plate was then washed three times with PBS and each well cut off and counted in a gamma counter set for <sup>125</sup>I.

#### *Assay for neutralization of TNF cytotoxicity*

TNF cytotoxicity was assayed as previously described (Aderka *et al.*, 1989). Rhabdomyosarcoma A673/6 cells were seeded in a 96-well plate at  $3 \times 10^4$  cells/well in Dulbecco's modified Eagle's medium with 10% fetal bovine serum about 16 hr prior to beginning the assay. Serial dilutions of the antibodies to be tested for neutralization were prepared in medium containing 40  $\mu$ g/ml of cycloheximide, and an equal volume of 80  $\mu$ g/ml recombinant (Dainippon) or natural (Genzyme, Inc.) TNF in medium was added to each dilution. The samples were incubated for 20-30 min at room temperature, then 100  $\mu$ l was dispensed into 96-well plates in duplicate. The plate was incubated at 39°C for 18-24 hr, after which each well was rinsed once with PBS. The adherent cells were fixed by adding 50  $\mu$ l/well of 10% formalin in PBS for 15 min at room temperature, and were stained with 50  $\mu$ l/well of 0.5% naphthol blue black in 9% acetic acid, 0.1 M sodium acetate. After 30 min at room temperature, the cells were rinsed with distilled water, and the bound dye was eluted with 150  $\mu$ l/well of 50 mM NaOH. Absorbance of the eluted dye was determined at 630 nm.

## RESULTS

#### *Cloning of the TNF-specific variable gene regions*

For the heavy chain, positive clones were isolated from the phage library made from 7.5 kb Eco RI fragments. The clones were mapped with restriction endonucleases and shown to be identical and consistent with a rearranged heavy chain locus. DNA sequence analysis demonstrated the existence of an open reading frame with homology to previously described heavy chain variable regions (data not shown). One variable region fragment was subcloned into an appropriate expression vector, and was shown to direct the synthesis and secretion of an antibody when coexpressed with an irrelevant cloned light chain gene. A restriction map of the heavy chain gene is shown in Fig. 1A.

For the light chain, several positive clones were obtained for each size class of Hind III fragments defined by the initial Southern blot. To identify the correct A2

## Characterization of a mouse-human chimeric anti-TNF antibody

1447

light chain gene from among the various possibilities, a combination of methods was employed. The authentic A2 light chain gene must have the following characteristics: (1) it must exhibit a restriction enzyme map consistent with a rearranged kappa locus; (2) it must hybridize with mRNA expressed in the murine A2 hybridoma; (3) its DNA sequence must contain an appropriate open reading frame to code for an immunoglobulin light chain; (4) it must direct the synthesis of a kappa light chain after incorporation into a suitable expression vector and transfection into an appropriate cell line; and (5) the light chain gene must give rise to a high-affinity anti-TNF antibody when coexpressed with the A2 heavy chain gene.

A representative of the 6 kb size class of *Hind* III fragments was ruled out because restriction maps and DNA sequence analysis indicated it was unrelated to the kappa locus. A clone (designated 4.0) containing a 4.0 kb *Hind* III fragment met all of the above criteria except the last. Although an antibody was secreted after coexpression of this light chain gene with the chimeric A2 heavy chain gene, the resulting antibody did not bind recombinant human TNF in a solid ELISA assay, suggesting that although the gene coded for a functionally expressed kappa light chain, it was of the wrong specificity. A partial DNA sequence was obtained for clone 4.0 and was found to be very similar to a light chain derived from the MPC11 murine myeloma. Two different classes of clones were obtained from the 3 kb size fractionated *Hind* III fragments. Both types hybridized to the appropriate size mRNA from the A2 hybridoma upon Northern analysis and had restriction maps consistent with a rearranged kappa locus. The maps were similar to each other but had distinct differences when analysed on a fine-structure level. DNA sequence analysis indicated that one type of clone (exemplified by clone 4.3) contained an appropriate reading frame coding for a kappa light chain. Another type (exemplified by clone 8.3) had a very similar sequence but contained differences resulting in frameshifts, amino acid changes and deletions compared to clone 4.3. Both 4.3 and 8.3 *Hind* III fragments were approximately 2.9 kb in length. Figure 1B highlights the sequence differences observed between clones 4.3 and 8.3. Interestingly, although there are multiple differences throughout the coding region, the 5' flanking regions are nearly identical for approximately 1 kb upstream of the coding region. Clone 4.3 was subsequently shown to be correct as it was able to express a chimeric light chain which, when combined with the chimeric A2 heavy chain, yielded an antibody of high affinity and appropriate specificity for human TNF.

#### *Expression of chimeric A2 antibody*

The putative light and heavy chain variable region (*V*) genes cloned from the A2 hybridoma were joined to human kappa and G1 constant region genes, respectively, in expression vectors previously described (Sun *et al.*, 1987). The 7.5 kb *Eco* R I fragment corresponding to the putative heavy chain *V* region gene from A2 was

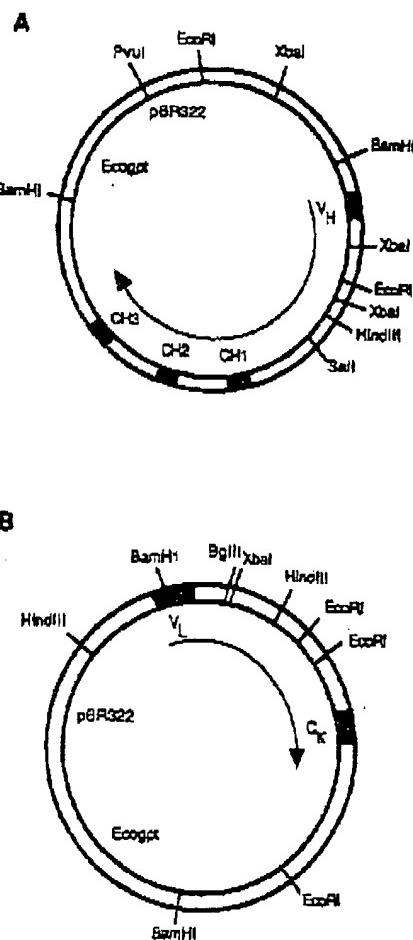


Fig. 2. Plasmids used for expression of the chimeric A2 heavy and light chain genes. Both heavy and light chain expression plasmids are derived from pSV2gpt (Mulligan and Berg, 1981), and contain the Ecogpt gene expressed from the SV40 early promoter, and pBR322 sequences. Transcription is driven by the natural promoters associated with the variable regions. A. Heavy chain expression plasmid pA2HG1apgt containing the A2 *V<sub>H</sub>* and human  $\gamma 1$  constant region exons. The arrow indicates the direction of transcription for the heavy chain gene. B. Light chain expression plasmid pA2HuKapgt containing the A2 *V<sub>L</sub>* and human *C<sub>K</sub>* exons. The arrow indicates the direction of light chain gene transcription.

cloned into an expression vector containing the human  $\gamma 1$  constant region and the Ecogpt gene to yield pA2HG1apgt. For the light chain, the 2.9 kb putative light chain fragment from clone 4.3 was subcloned into a vector containing the human kappa constant region and the Ecogpt gene. The resulting plasmid was designated pA2HuKapgt. The A2 expression plasmids are shown in Fig. 2.

To express the chimeric heavy and light chain genes, the expression plasmids were transfected into the non-producing mouse myeloma cell line SP2/0, and mycophenolic acid selection was applied after 48 hr.

Resistant colonies were expanded to stable cell lines and tissue culture supernatant from these cell lines were tested for antibody using an ELISA assay. The highest producers were subcloned, and a cell line was isolated that produces greater than 100 µg/ml chimeric antibody in 7-day static cultures when seeded at  $1 \times 10^3$  cells/ml.

#### *Characterization of chimeric A2 antibody*

The chimeric A2 antibody was purified by adjusting tissue culture supernatant to 0.1 M Tris, 0.002 M EDTA, pH 8.0 and loading on a Protein A-sepharose column equilibrated in the same buffer. The IgG was eluted with 0.1 M citrate pH 3.5, neutralized with 1 M Tris and dialysed against PBS.

The purified chimeric antibody was evaluated for its ability to bind to purified human recombinant TNF in a solid-phase ELISA assay. Figure 3 demonstrates that both murine and chimeric A2 (cA2) bind to rTNF, and that cA2 appears to bind somewhat better at low Ab concentration than murine A2, although direct comparison is difficult because different secondary antibodies were used.

For a direct binding comparison and to confirm that the murine and chimeric antibodies recognize the same binding site on TNF, a competition assay was used in a

solid-phase format. A constant amount of  $^{125}\text{I}$ -labeled A2 or cA2 was mixed with varying amounts of unlabeled competitor and radioactivity bound to rTNF was measured. The results presented in Fig. 4 indicate that cA2 and murine A2 compete with each other for binding to rTNF. The competition experiments also suggest that cA2 is a better competitor for either  $^{125}\text{I}$ -chimeric or  $^{125}\text{I}$ -murine A2 than is murine A2. This result is consistent with the data obtained in the ELISA assay (see above).

Because the binding of cA2 appeared to be better than that of the murine antibody, we wished to quantitate this effect by Scatchard analysis using data obtained from a solid-phase RIA assay; the results of one experiment for each antibody are shown in Fig. 5. Relative association constants were calculated averaging the results from two such experiments, each performed in triplicate, yielding a  $K_a = 5.0 \times 10^9 \text{ M}^{-1}$  for the murine A2 and  $K_a = 1.8 \times 10^9 \text{ M}^{-1}$  for cA2, thus confirming the improved binding of the chimeric antibody observed in previous assays. It is likely that the difference in relative affinity constants reflects the fact that the murine hybridoma expresses two different kappa light chains, only one of which is the "correct" anti-TNF light chain. The other light chain is derived from the 4.0 kb *Hind* III

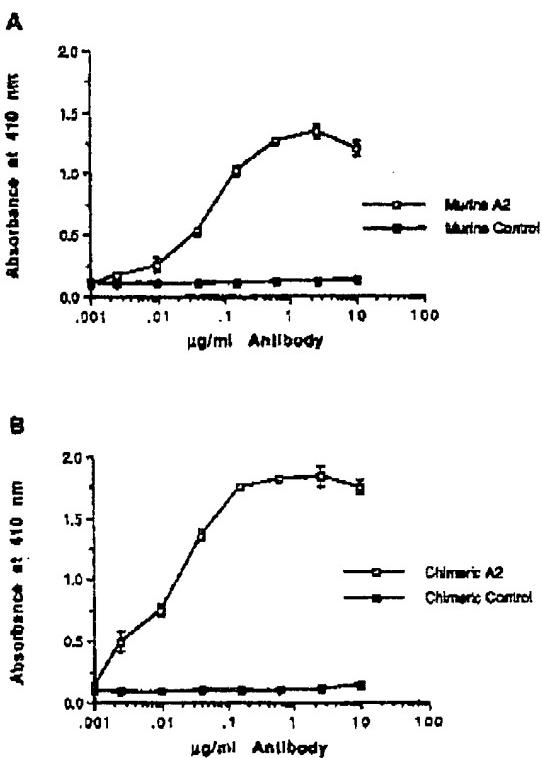


Fig. 3. Binding of murine and chimeric A2 antibodies to recombinant human TNF. rTNF was immobilized on polystyrene plates, purified murine or chimeric A2 was added, and bound antibody was detected with alkaline phosphatase-conjugated goat anti-murine or anti-human reagents as described in Materials and Methods. A. Murine A2. B. Chimeric A2. Isotype-matched negative control antibodies are also shown.

## Characterization of a mouse-human chimeric anti-TNF antibody

1449

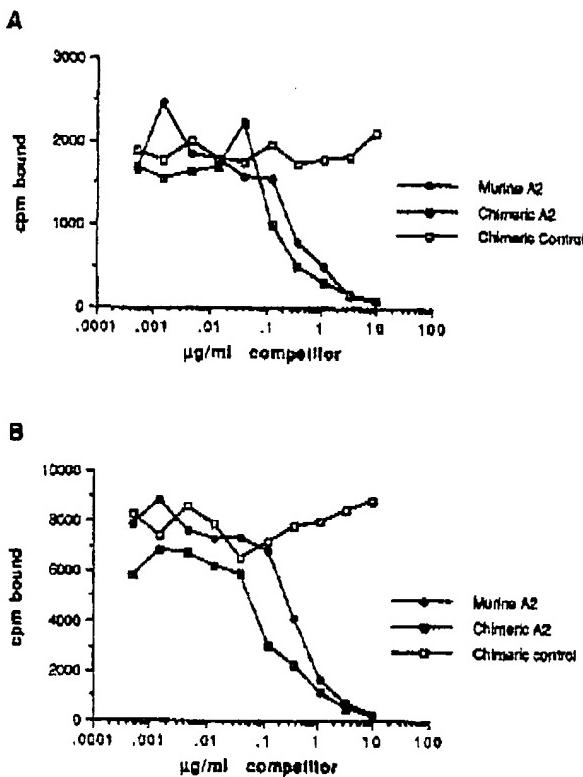


Fig. 4. Competitive inhibition assay comparing the ability of unlabeled murine or chimeric antibodies to compete for binding to rTNF with  $^{125}\text{I}$ -labeled A2 or cA2. A constant amount of  $^{125}\text{I}$ -labeled murine A2 (A) or chimeric A2 (B) was incubated with various amounts of unlabeled competitor antibody as indicated. After washing, bound  $^{125}\text{I}$  was determined by gamma counting as described in Materials and Methods. An irrelevant chimeric  $\gamma 1$  antibody is also shown as a negative control.

fragment cloned from the A2 hybridoma which we showed was capable of expressing a kappa protein. Because the antibody derived from the murine hybridoma contained a mixture of the correct and incorrect light chains, it exhibits a lower apparent affinity than the antibody from the transfected cell line which produces only the correct light chain. Support for this hypothesis is provided by N-terminal protein sequence analysis of the murine A2 light chain. A mixture of two sequences was obtained that is consistent with the deduced sequences of the two functional genomic clones obtained (data not shown).

The murine and chimeric A2 antibodies were compared in a functional assay which measures neutralization of TNF activity. TNF exhibits cytotoxicity towards the rhabdomyosarcoma cell line A673/6; the capacity of the antibodies to neutralize this TNF-dependent cytotoxicity is shown in Fig. 6. Both chimeric and murine A2 were able to neutralize the effect of recombinant TNF (Fig. 6A) and natural TNF (Fig. 6B), with the chimeric proving to be slightly more potent in both cases. Neither antibody was capable of neutralizing human lymphotoxin (TNF- $\beta$ ) demonstrating their specificity for TNF- $\alpha$  (data not shown).

## DISCUSSION

Neutralization of TNF- $\alpha$  action has been suggested as a mode of therapeutic intervention in a variety of human diseases as a result of emerging data implicating TNF as an important pathophysiological regulator. Elevated TNF levels are associated with cachexia, septic shock resulting from acute bacterial infection and autoimmune diseases. Monoclonal antibodies are well suited for the task of neutralizing and/or clearing from circulation harmful soluble mediators. Accordingly, we have generated a murine hybridoma that secretes an anti-human TNF monoclonal antibody (A2) with high affinity for TNF and ability to neutralize its various biological activities. The A2 antibody neutralizes TNF- $\alpha$ , but not the related cytokine TNF- $\beta$ , and reacts with both recombinant and natural human TNF. The antibody has the ability to neutralize TNF $\alpha$  from human and chimpanzee, but not from baboon, cynomolgus or rhesus monkeys, dog, pig, rabbit, rat or mouse (data not shown). To reduce the potential immune response which may limit repetitive use and to optimize functional compatibility with the human immune system, we have prepared a recombinant version of the A2 antibody by replacing the murine constant regions with human counterparts while

retaining the antigen binding region of the original antibody. Many mouse-human antibodies have been constructed (Sun *et al.*, 1987; Morrison *et al.*, 1984; Boulianne *et al.*, 1984; Sahagan *et al.*, 1986; Nishimura *et al.*, 1987; Heinrich *et al.*, 1989) and the few that have been tested in humans display various degrees of immunogenicity, presumably due to the range of immune responses elicited by the variable regions of the individual antibodies (LoBuglio *et al.*, 1989; Meredith *et al.*, 1991; Saleh *et al.*, 1992; Khazaeli *et al.*, 1992). When direct comparisons of murine and chimeric versions of an antibody have been made in humans, however, the immunogenicity of the chimeric antibody has generally been lower than that of the murine antibody (LoBuglio *et al.*, 1989; Meredith *et al.*, 1991; Saleh *et al.*, 1992). Antibodies with human constant domains may also be more efficient in Fc region-mediated effector functions such as complement fixation, antibody-dependent cellular cytotoxicity and Fc-mediated antibody clearance. An increased circulating half-life has also been demon-

strated for chimeric antibodies, a desirable feature for prolonged therapeutic effect [for review see Larrick and Fry (1991)].

The construction of the chimeric A2 antibody resulted in an antibody with improved binding and neutralizing characteristics compared to the original antibody isolated from the A2 murine hybridoma. Presumably this is a consequence of multiple kappa light chain expression in the hybridoma; we have shown that at least two different genes cloned from the hybridoma are capable of expressing kappa chains, but that only one of them gives rise to an anti-TNF antibody when paired with the A2 chimeric heavy chain. N-terminal sequence analysis of the murine mAb confirmed the presence of two different light chains; the irrelevant light chain (clone 4.0) is likely to be responsible for the lower apparent affinity of the murine mAb compared to cA2. The gene for the irrelevant light chain was apparently contributed by the fusion partner of the A2 hybridoma. Southern analysis shows that the 4.0 kb *Hind* III fragment is present in both the fusion partner and the A2 hybridoma, and cDNA was prepared from the fusion partner mRNA which has the same coding sequence as clone 4.0 from the hybridoma (data not shown). This sequence, when compared to the Genbank DNA sequence database, was found to be very similar to a light chain derived from the MPC11 murine myeloma (Kuchi and Scharff, 1974). The SP2/0 cell line used for cell fusion was from a different source than the SP2/0 cells used to express the chimeric antibody. It is unclear why the particular SP2/0 line used for cell fusion expresses the irrelevant light chain. The SP2/0 cell line has been reported to express a kappa mRNA from an aberrantly rearranged gene that does not produce a functional kappa light chain (Carroll *et al.*, 1988). None of the kappa clones isolated from the A2 hybridoma contain the sequence found for the SP2/0 aberrant transcript.

In addition, a third kappa light chain gene (clone 8.3) was cloned from the hybridoma which has extensive homology to the authentic A2 V kappa gene, although it cannot be expressed as a functional protein. Clones 4.3 and 8.3 appear to result from independent V-J recombination events; the 8.3 V-J junction causes a shift in the reading frame, and three consecutive nucleotides differ from the 4.3 productive rearrangement at the junction. The nearly identical sequences upstream of the V regions suggest that two different alleles of the same V region gene each independently rearranged to a J4 minigene. One rearrangement was nonproductive (8.3) and one was productive (4.3). Presumably, each V region underwent localized somatic hypermutation during B-cell maturation (French *et al.*, 1989). This may explain the many sequence differences between 8.3 and 4.3 in and around the V-J regions. The observed patterns of sequence differences are consistent with the boundaries of somatic mutation for rearranged immunoglobulin genes previously reported (Lebecque and Gearhart, 1990). Although the precise origins of the genes represented by clones 4.3 and 8.3 are unclear, their coexistence in a single cloned hybridoma line complicated the cloning

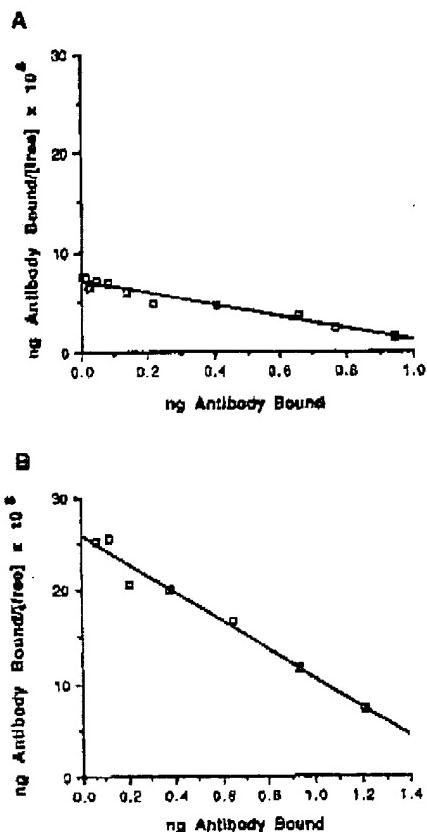


Fig. 5. Scatchard analysis to determine relative association constants for murine and chimeric A2 antibodies. The assay was performed in a solid-phase format with immobilized rTNF and <sup>125</sup>I-labeled antibodies as described in Materials and Methods. The results were plotted for murine A2 (A) and chimeric A2 (B) as nanograms (ng) of antibody bound vs ng bound/unbound antibody, and the slope is the relative association constant ( $K_a$ ) for the antibody.

## Characterization of a mouse-human chimeric anti-TNF antibody

1451

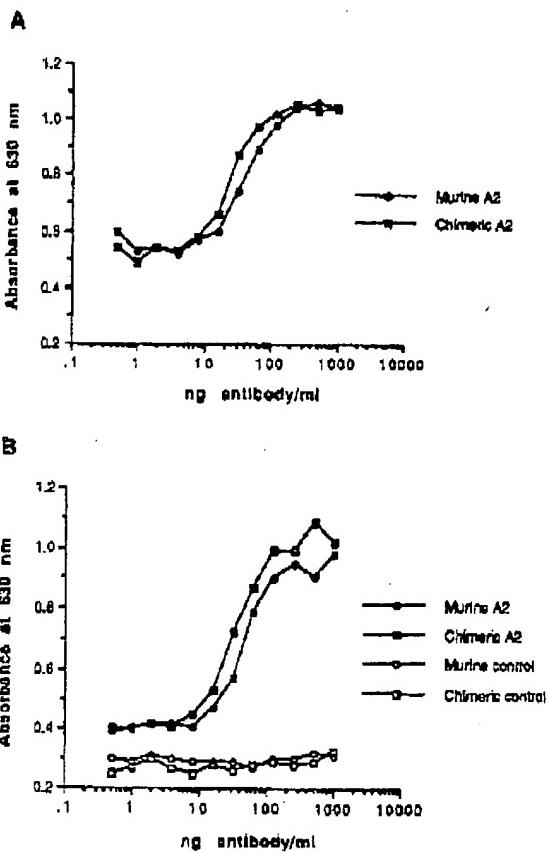


Fig. 6. Neutralization of TNF-mediated cytotoxicity by murine and chimeric A2 antibodies. The cytotoxic effect of recombinant (A) or natural (B) TNF on rhabdomyosarcoma A673/6 cells was measured in the presence of various amounts of murine or chimeric A2 as described in Materials and Methods. The results with isotype-matched negative control antibodies are shown for the natural TNF cytotoxicity experiment.

and analysis of the correct A2 light chain gene. A fourth type of clone was isolated that hybridized to the  $J_\kappa$  probe, but was not characterized further after restriction enzyme mapping indicated it was not a normal kappa gene. Of the four genes cloned from the A2 hybridoma using the light chain probe, two have been shown to be expressed as kappa proteins, and at least three and possibly four are expressed as mRNA. The expression of two kappa proteins in a single hybridoma line has implications beyond the technical cloning problems encountered. As we have shown, the antibody isolated from a hybridoma can be a mixture of different chains, thus diluting the effectiveness of the specific antibody. The expression of multiple antibody genes may be fairly common in hybridomas, and may not be readily apparent unless protein sequencing or molecular cloning is performed. In the case of the A2 antibody, the cloning of the correct genes and expression in chimeric form in the appropriate cell line resulted in a reagent with superior binding characteristics compared to the mixture expressed by the A2 hybridoma.

There is considerable potential for the therapeutic use of anti-TNF antibodies in many diseases. Preliminary studies with murine anti-TNF monoclonal antibodies given to septic patients (Exley *et al.*, 1990; Spooner *et al.*, 1991) report a high incidence of human anti-mouse antibody (HAMA) response. Use of the chimeric A2 anti-TNF antibody should lead to reduced HAMA response and improved pharmacokinetics, thus providing significant advantages over murine antibodies, especially in chronic diseases such as rheumatoid arthritis which may require multiple treatments. A Phase I clinical trial of cA2 in patients with severe rheumatoid arthritis showed dramatic clinical improvement in all patients (Elliott *et al.*, 1993), indicating that this antibody has great promise as a therapeutic agent.

## REFERENCES

- Aderka D., Le J. and Vilcek J. (1989) IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells and in mice. *J. Immunol.* 143, 3517-3523.

- Beuller B. and Cerami A. (1988) Tumor necrosis, cachexia, shock and inflammation: a common mediator. *A. Rev. Biochem.* 57, 505-518.
- Boulianne G., Hozumi N. and Shulman J. (1984) Production of functional chimeric mouse/human antibody. *Nature* 312, 643-646.
- Brennan F. M., Chantry D., Jackson A. M., Maini R. N. and Feldmann M. (1989a) Cytokine production in culture by cells isolated from the synovial membrane. *J. Autoimmun.* 2 (Suppl.), 177-186.
- Brennan F. M., Chantry D., Jackson A., Maini R. and Feldmann M. (1989b). Inhibitory effect of TNF $\alpha$  antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. *The Lancet* 2, 244-247.
- Brennan F. M., Field M., Chu C. Q., Feldmann M. and Maini R. N. (1991) Cytokine expression in rheumatoid arthritis. *Br. J. Rheumatol.* 30 (Suppl. 1), 76-80.
- Carroll W. L., Mendel E. and Levy S. (1988) Hybridoma fusion cell lines contain an aberrant kappa transcript. *Molec. Immunol.* 25, 991-995.
- Cope A. P., Aderka D., Doherty M., Engelmann H., Gibbons D., Jones A. C., Brennan F. M., Maini R. N., Wallach D. and Feldmann M. (1992) Increased levels of soluble tumor necrosis factor receptors in the sera and synovial fluid of patients with rheumatic diseases. *Arth. Rheum.* 35, 1100-1109.
- Deleuron B. W., Chu C. Q., Field M., Brennan F. M., Mitchell T., Feldmann M. and Maini R. N. (1992) Localization of tumor necrosis factor receptors in the synovial tissue and cartilage-pannus junction in patients with rheumatoid arthritis. *Arth. Rheum.* 35, 1170-1178.
- Elliott M. J., Maini R. N., Feldmann M., Williams R. O., Brennan F. M. and Chu C. Q. (1993) Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to TNF $\alpha$ : safety, clinical efficacy and control of the acute-phase response. *J. cell. Biochem.* (Suppl.) 17B, 145.
- Efrey A. R., Cohen J., Bouman W., Owen R., Hanson G., Lumley J., Auliock J. M., Bodmer M., Riddell A., Stephens S. and Perry M. (1990) Monoclonal antibody to TNF in severe septic shock. *Lancet* 335, 1275-1277.
- French D. L., Laskov R. and Scharff M. D. (1989) The role of somatic hypermutation in the generation of antibody diversity. *Science* 244, 1152-1157.
- Freudenberg M. A. and Galanos C. (1991) Tumor necrosis factor alpha mediates lethal activity of killed gram-negative and gram-positive bacteria in D-Galactosamine-treated mice. *Infect. Immun.* 59, 2110-2115.
- Heinrich G., Gram H., Kocher H., Schreier M., Ryffell B., Akbar A., Amiot P. and Janossy G. (1989) Characterization of a human T cell-specific chimeric antibody (CD7) with human constant and mouse variable regions. *J. Immunol.* 143, 3589-3597.
- Hinshaw L. B., Tekamp-Olson P., Chang A. C. K., Let P. A., Taylor F. B., Murray C. K., Peer G. T., Emerson T. E., Passey R. B. and Kuo G. C. (1990) Survival of primates in LD<sub>50</sub> septic shock following therapy with antibody to tumor necrosis factor (TNF $\alpha$ ). *Circ. Shock* 30, 279-292.
- Hofman F. M., Hinton D. R., Johnson K. and Merrill J. E. (1989) Tumor necrosis factor identified in multiple sclerosis brain. *J. exp. Med.* 170, 607-612.
- Jacob C. O. and McDevitt H. O. (1988) Tumor necrosis factor  $\alpha$  in murine autoimmune lupus nephritis. *Nature* 331, 356-358.
- Khazaie M. B., Saleh M. N., Liu T., Kaladas P. M., Gilman S. C. and LoBuglio A. F. (1992) Frequencies of anti-variable region immune responses to mouse B72.3 monoclonal antibody. *J. Clin. Immunol.* 12, 116-121.
- Kuchi W. M. and Scharff M. D. (1974) Synthesis of a carboxyl-terminal (constant region) fragment of the immunoglobulin light chain by a mouse myeloma cell line. *J. molec. Biol.* 89, 409-421.
- Lerrick J. W. and Fry K. E. (1991) Recombinant antibodies. *Hum. Antibod. Hybridomas* 2, 172-189.
- Lebecque S. G. and Gearhart P. J. (1990) Boundaries of somatic mutation in rearranged immunoglobulin genes: S'-boundary is near the promotor, and J'-boundary is ~ 1 kb from V (D) J gene. *J. exp. Med.* 172, 1717-1727.
- Leist T. P., Frei K., Kam-Hansen S., Zinkernagel R. M. and Fontana A. (1988) Tumor necrosis factor  $\alpha$  in cerebrospinal fluid during bacterial, but not viral meningitis. *J. exp. Med.* 167, 1743-1748.
- LoBuglio A. F., Wheeler R. H., Trang J., Haynes A., Rogers K., Harvey E. B., Sun L., Ghayeb J. and Khazaie M. B. (1989) Mouse/human chimeric monoclonal antibody in man: kinetics and immune response. *Proc. natn. Acad. Sci. U.S.A.* 86, 4220-4224.
- Meredith R. F., Khazaie M. B., Plott W. E., Saleh M. N., Liu T., Allen L. F., Russel C. D., Orr R. A., Colcher D., Schlom J., Shochat D., Wheeler R. H. and LoBuglio A. F. (1991) Phase I trial of Iodine-131-Chimeric B72.3 (Human IgG4) in metastatic colorectal cancer. *J. Nucl. Med.* 33, 23-29.
- Morrison S., Johnson M., Herzenberg L. and Oi V. (1984) Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc. natn. Acad. Sci. U.S.A.* 81, 651-655.
- Mulligan R. C. and Berg P. (1981) Factors governing the expression of a bacterial gene in mammalian cells. *Molec. cell. Biol.* 1, 449-459.
- Nishimura Y., Yokoyama M., Araki K., Ueda R., Kudo A. and Watanabe T. (1987) Recombinant human-mouse chimeric monoclonal antibody specific for common acute lymphocytic leukemia antigen. *Cancer Res.* 47, 999-1005.
- Opal S. M., Gross A. S., Kelly N. M., Sadoff J. C., Bodmer M. W., Palardy J. E. and Victor G. H. (1990) Efficacy of a monoclonal antibody directed against tumor necrosis factor in protecting neutropenic rats from lethal infection with *Pseudomonas aeruginosa*. *J. Infect. Dis.* 161, 1148-1152.
- Sahagan B. G., Dorai H., Muller J. S., Toneguzzo F., Guindon C. A., Lilly S. P., McDonald K. W., Morrissey D. V., Stone B. A. and Davis G. L. (1986) A genetically engineered murine/human chimeric antibody retains specificity for human tumor-associated antigen. *J. Immunol.* 137, 1066-1074.
- Saleh M. N., Khazaie M. B., Wheeler R. H., Allen L., Tilden A. B., Grizzle W., Reisfeld R. A., Yu A. L., Gillies S. D. and LoBuglio A. F. (1992) Phase I trial of the chimeric anti-GD2 monoclonal antibody ch14.18 in patients with malignant melanoma. *Hum. Antibod. Hybridomas* 3, 19-24.
- Shalaby M. R., Fendly B., Sheehan K. C., Schreiber R. D. and Ammann A. J. (1989) Prevention of the graft-versus-host reaction in newborn mice by antibodies to tumor necrosis factor- $\alpha$ . *Transplantation* 47, 1057-1061.
- Shimamoto Y., Chen R. L., Bollon A., Chang A. and Khan A. (1988) Monoclonal antibodies against human recombinant tumor necrosis factor: prevention of endotoxic shock. *Immunol. Lett.* 17, 311-318.
- Silva A. T., Bayston K. F. and Cohen J. (1990) Prophylactic and therapeutic effects of a monoclonal antibody to tumor necrosis factor- $\alpha$  in experimental gram-negative shock. *J. Infect. Dis.* 162, 421-427.

## Characterization of a mouse-human chimeric anti-TNF antibody

1453

- Spooer C., Saravolatz L., Markowitz N., Allred R., Wherry J. and Pennington J. (1991) Safety and pharmacokinetics of murine monoclonal antibody to human tumor necrosis factor (TNF-Mab). Abstract in ICAAC 31st interscience conference on anti-microbial agents and chemotherapy, Abstract # 341, p. 189. ASM Press.
- Sun L. K., Curtis P., Rakowicz-Szulczynska E., Ghayeb J., Chang N., Morrison S. L. and Koprowski H. (1987) Chimeric antibody with human constant regions and mouse variable regions directed against carcinoma-associated antigen 17-1A. *Proc. natn. Acad. Sci. U.S.A.* **84**, 214-218.
- Tracey K. and Cerami A. (1989) Cachectin/tumor necrosis factor and other cytokines in infectious disease. *Curr. Opin. Immunol.* **1**, 454-461.
- Tracey K., Fong Y., Hesse D. G., Manogue K. R., Lee A. T., Kuo G. C., Lowry S. F. and Cerami A. (1987) Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature* **330**, 662-664.
- Williams R. O., Feldmann M. and Maini R. N. (1992) Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc. natn. Acad. Sci. U.S.A.* **89**, 9748-9752.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER: \_\_\_\_\_**

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**